REMARKS

Claims 21-32 are pending in this application. Claims 21-32 are amended herein for clarity to more particularly describe the invention. Support for these amendments can be found in the original claim language and throughout the specification, as set forth below. It is believed that these amendments add no new matter. In light of these amendments and the following remarks, applicant respectfully requests reconsideration of this application, entry of these amendments, and allowance of the claims.

Attached hereto is a marked up version of the changes made to the claims by the current amendment. The attachment is captioned **"VERSION WITH MARKINGS TO SHOW CHANGES MADE."**

Rejections under 35 U.S.C. § 101

Claims 25 and 27 are rejected under 35 U.S.C. § 101 as allegedly being drawn to non-statutory matter. Specifically, the Office Action states that the term "host" is viewed as encompassing humans, thus encompassing non-statutory matter. The Examiner suggests amending the claims by deleting the term "host" and substituting therefor the phrase "an isolated cell."

Claims 25 and 27 are amended according to the Examiner's suggestion. The term "host" is deleted, and the phrase "an isolated cell" is substituted therefor in each claim. Applicant believes that these rejections are overcome and respectfully request that these rejections be withdrawn and that amended claims 25 and 27 be allowed. Moreover, dependent claims 26 and 28 are amended herein to conform to the language of claims 25 and 27, thereby obviating a rejection under 35 U.S.C. § 101.

Double Patenting Rejections

Claims 25-28 are rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 11 and 12 of U.S. Patent No. 5,455,169. The Office Action states that although the conflicting claims are not identical, they are not patentably distinct from each other because the specific nucleic acid sequences claimed in claims 11 and 12 of the '169 patent are encompassed within the scope of claims 25-28 of the pending application.

Applicant will either file a terminal disclaimer or cancel claims 25-28 upon receipt of a Notice of Allowability.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 21-24, 29 and 30 are rejected under 35 U.S.C.§ 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Further, claims 25-28, 31 and 32 are rejected under 35 U.S.C.§ 112, first paragraph, allegedly because the specification, while being enabling for isolated cells which comprise and express a nucleic acid sequence encoding human amyloid precursor protein APP 770 operably linked to a promoter, wherein the nucleic acid encodes an amino acid 670 other than lysine and/or at amino acid 671 other than methionine, and further encodes other than valine at amino acid 717, such that detectable levels of said APP 770 are produced by the cells, and methods of screening using the cell lines, does not reasonably provide enablement for transgenic non-human mammals, hosts in the broadest definition by the specification. The Office Action goes on to state that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Claims 25 and 27 are amended herein, deleting the term "host" and substituting therefor the phrase "an isolated cell." Moreover, dependent claims 26, 28, 31 and 32 are amended herein to

conform to the language of amended claims 25 and 27. Thus, applicant believes that these rejections are overcome and respectfully request that these rejections be withdrawn and that amended claims 25-28, 31 and 32 be allowed.

The Office Action states that for claims 21-32 to be enabled, the non-human transgenic animal host or the non-human transgenic animal must be readily available to the public from a reproducible source for animal, animal host or methods of screening. The Office Action goes on to allege that the specification does not provide either a source for the transgenic non-human animal or an enabling method of making such animal. Applicant respectfully traverses this rejection.

The standard for making a rejection based on 35 U.S.C. § 112, first paragraph, is articulated in *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988) (see also MPEP § 2164.01 and 2164.04). Initially, the Office must accept the objective truth of statements made in the specification. If such statements are to be called into question, the Office is burdened with providing evidence or convincing argument why those of skill in the art would doubt the statements (*In re* Marzocchi, 439 F.2d 220, 169 USPQ 367 (CCPA 1971)). Applicant asserts that this burden has not been met. The objective truth of the statements in the specification regarding making of the claimed transgenic non-human animals is to be accepted unless evidence or convincing reasoning can be provided by the Office. The office has not applied this standard as described below.

The Office Action alleges that those of skill in the art, based on the teachings of the specification, would not be able to reproducibly make a transgenic animal, i.e., mouse, that

expresses the transgene and manifests the neurological pathologies associated with Alzheimer's Disease. In fact, the application teaches the steps that would be consistently used for making a transgenic mouse that manifests neuropathologic signs of Alzheimer's Disease. Furthermore, applicant notes that there is no requirement that the claimed transgenic non-human animal be literally demonstrated by the production of such an animal. See *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ2d 1302, 1304 (Fed. Cir. 1987) (holding that the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it). Moreover, "[c]ompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, does not turn on whether an example is disclosed." M.P.E.P. § 2164.02. In fact, "[t]he specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation." See *In re Borkowski*, 422 F.2d 904, 908 (CCPA 1970). For all of these reasons, laid out in more detail below, applicant submits that no proper *prima facie* case for lack of enablement has been established.

The Office Action states that the specification discloses that the transgene can be injected into mouse embryos and that the transgene can be identified to be integrated into the mouse genome. The Office Action alleges that applicant has not provided guidance as to which of the several constructs described on pages 23-25 will produce transgenic non-human animals or transgenic mice that express the integrated transgene to a level that the animals or the mouse can be used in a screening assay or otherwise as an Alzheimer's Disease model. The Office Action alleges that applicant has not shown that the microinjection method reproducibly produces transgenic animals or mice that express the transgene so as to alter the phenotype of the animal or

mouse such that it can serve as a model for Alzheimer's Disease. The Office Action further alleges that there is no evidence of record that the mice injected with one of applicant's disclosed transgenes will develop a symptom or characteristic of Alzheimer's Disease, such as senile plaques, neurofibrillary tangles, neuritic processes and neuronal loss in the brain. The Office Action states that the specification does not disclose mice that have these characteristics, and thus the specification does not enable a mouse as a model for use as a screening assay for Alzheimer's Disease. The Office Action concludes that "without a showing of symptom(s) or characteristic(s) of Alzheimer's Disease, the mouse as disclosed would not have an enabled use as a AD model for use as a screening assay. The specification does not show such, and therefore the disclosed mice have not been shown to have neuropathological characteristics associated with Alzheimer's Disease."

The application does much more than merely state that a transgene can be injected into an embryo and integrated into the mouse genome. Applicant teaches how to make and use transgenic constructs that contain a mutant APP nucleotide sequence operably linked to a promoter, for example neural-specific enolase (NSE), that controls expression of the mutated sequence in the non-human animal. These transgenes encode a mutant APP having the Swedish mutation. See in the specification, page 23, line 8 through page 25, line 26. A person of skill at the time of the filing of the application could make these constructs using the teachings of the application. See in the specification page 23, line 8 through page 25, line 26. Further, applicant teaches how to prepare the DNA from the corresponding vector for injection into the non-human animal recipient. See page 25, lines 28-32. More specifically, applicant teaches how to deliver

the transgene into a recipient one-cell embryo using methods accepted in the art. The specification cites to the teaching of Hogan *et al.*, Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1986), a reference that enables the microinjection of genetic material into non-human zygotes. See page 25, line 34 through page 26, line 6. Further, applicant teaches that after overnight incubation, embryos that have divided into the two-cell stage can be implanted into pseudo-pregnant female mice and weaned at approximately 21 days. Samples of DNA obtained from tail biopsy can be analyzed by Southern blot, and transgenic mice containing at least one copy of the transgene can be identified. Thus, the application teaches the steps required to make the claimed transgenic animal. These steps were recognized in the art to produce transgenic animals.

An example of the routine nature of transgenic animal technology at the filing date of the present application, U.S. Patent No. 4,873,191, issued to Wagner *et al.*, October 10, 1989, discloses a method of making a transgenic animal. Specifically, the Example teaches a procedure for delivering exogenous genetic material into a pronucleus of a mammalian zygote by microinjection, and in claim 1 recites:

"A method of obtaining a mammal characterized as having a plurality of cells containing exogenous genetic material, said material including at least one gene and a control sequence operably associated therewith, which, under predetermined conditions, express said gene under the control of said control sequence in a cell of said mammal, which comprises:

(a) introducing exogenous genetic material into a pronucleus of a mammalian zygote by microinjection, said zygote being capable of development into a mammal, said genetic material including at least one gene and a control sequence operably associated therewith, thereby obtaining a genetically transformed zygote;

(b) transplanting an embryo derived from the genetically transformed zygote into a pseudo-pregnant female capable of bearing the embryo to term; and

(c) allowing the embryo to develop to term;

where said gene and control sequence are selected so that the gene is not activated in such manner and degree as would prevent normal development of the embryo to term."

The '191 patent (which has a presumption of validity) was issued nearly three years prior to the filing of the instant application, and its teachings were well known in the art. As a further example of the routine nature of making a transgenic mouse that manifests neuropathologic signs of AD, applicant submits Quon *et al.*, (*Nature*, vol. 352, pp. 239-241 1991, attached as Exhibit A). This reference was published and known to persons of skill in the art about one year before the filing of the instant application. These authors constructed a chimeric gene comprising human APP 751 and rat neural-specific enolase (NSE) and expressed the construct in mice, resulting in the deposition of β-amyloid deposits in the cortex and hippocampal brain regions of the transgenic mice that distinguished the transgenic mice from control mice. Thus, at the time of filing of the present application, it was known in the art how to make and use a transgenic mouse that expresses a human APP. Based on Quon *et al.*, it was also known in the art, prior to the present application, that NSE was effective to drive expression of APP in the brain. The present application teaches a construct comprising a mutant APP751 and a neural-specific

enolase (NSE) promoter. Because the steps for making these animals are essentially the same, regardless of the APP isoform, a transgenic animal containing the NSE promoter and a mutant APP 770 gene could be made without undue experimentation and reasonably expected to have a substantial and specific use. See in the specification page 23, lines 3-6. Therefore, using the teachings of the instant specification, a person of skill at the time of filing, could have made a transgenic mouse that expresses the mutant APP 770 and manifests β- amyloid deposits in the brain.

Further evidence of the state of the art of making transgenic animals at the time the instant application was filed, is found in U.S. Patent No. 5,387,742, which was filed on June 17, 1991 (about one year before the filing date of the instant application) and issued to Cordell *et al.* in 1995. In the '742 patent, the patentee teaches how to make and use two strains of transgenic mice, one strain whose cells contain a DNA sequence, comprising a nerve tissue specific promoter (rat NSE) operably linked to a DNA sequence that encodes APP 751, and the other strain whose cells contain a DNA sequence, comprising rat NSE operably linked to a DNA sequence that encodes APP 770. Each DNA construct is integrated in the genome of the mice and expressed to form β-amyloid deposits in the brain of the mice. See col. 36, lines 25-36. The '742 patent is evidence that at least one year before the instant application was filed, persons of skill in the art had the knowledge to make transgenic mice that express an APP sequence that encodes APP and forms β-amyloid deposits in the brain of the mice without undue

experimentation. Specifically, constructs using NSE (as taught by applicant) were known to be expressed in brain. Moreover, the teaching of the '742 patent is presumed to be enabled.

Like Quon *et al.* and the '742 patent, the instant application teaches how to make a DNA construct containing a variety of promoters operably linked to a nucleotide sequence that encodes an APP. In the instant application, the APP sequence contains a mutation at codon 670 and/or codon 671 and may contain a mutation at codon 717. Further, the instant application discloses proven techniques (See, e.g., Quon *et al.*, the '742 patent and the '191 patent) for delivering the DNA construct to a fertilized mouse egg and for transplanting the early embryo to a pseudo-pregnant mouse for full-term gestation. Thus, it is clear that at the time of filing of the instant application, a person of skill, using the teaching of the instant application, would have reasonably expected to make the claimed transgenic mouse without undue experimentation. Thus, the claimed invention is enabled. That applicant's teaching is to be taken with the knowledge in the art (e.g., Hogan, Quon *et al.*, the '742 patent and the '191 patent) is made clear in *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991), wherein the court stated that "[a] patent need not teach, and preferably omits, what is well known in the art." It was unnecessary for applicant to disclose in the instant specification further details of how to make transgenes or how to use microinjection to introduce the transgene or how to make an animal that expresses the transgene in a useful way. Thus, a person of skill at the time of filing of the instant application could, without undue experimentation, select a DNA construct, prepare the DNA for injection, deliver the transgene

into a non-human animal recipient and thus make a transgenic animal that expresses a mutant APP.

As to which of the constructs will work, even if all of the constructs disclosed in the specification were made in order to produce a non-human transgenic animal, a person of skill, at the time of the filing of the application, would be able to produce such an animal without undue experimentation. In *In re Wands*, wherein the issue was the predictability of being able to make a particular monoclonal antibody, the court states that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue,' not experimentation." The court further states that "[t]he determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." Id. at 1404. In the transgenic animal art at the time this application was filed, the level of skill was high, and those persons of skill expected to perform a significant amount of experimentation to obtain the transgenic animal of interest. Thus, applying the standard of reasonableness as required by *In re Wands*, there is no

basis to assert that the experimentation that might be required to produce the claimed transgenic animal is undue.

With regard to the Office's allegation that the present invention does not enable a mouse that can be used in a screening assay or otherwise as an Alzheimer's Disease model, applicant respectfully points out that the Examiner errs when she assumes that the mouse of the claimed invention must necessarily manifest β-amyloid in the brain to have a use as a model for Alzheimer's Disease. While applicant establishes herein that such a model can be produced without undue experimentation, in fact, the claimed transgenic mouse can also be used to screen for compounds that affect amyloid protein processing. For example, U.S. Patent No. 5,720,936, issued to Wadsworth *et al.* in 1998, provides a transgenic mouse comprising a DNA construct containing an APP gene operably linked to one of several promoters (e.g., NSE (as taught in the instant specification), human β actin gene promoter, human platelet derived growth factor B chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter) that can be delivered to a fertilized mouse egg by microinjection techniques disclosed in the instant application. This transgenic mouse has the specific, substantial and credible use to screen for compounds that may affect the of production of APP and β-amyloid peptide in the animal. In this issued patent it is recognized by the Office that this utility is sufficient, because it is this screening method to which the issued claims are directed. It is clear, therefore, that the transgenic mouse of the instant application can also be

used in assays for compounds that can affect the amount of APP and β-amyloid peptide production in the animals. This is the case whether or not β-amyloid deposits are present. Thus, while applicant has established herein that the construct design would be expected to result in the desired brain expression of APP and deposition of β-amyloid plaques in the brain, it is also the case that plaques in the brain per se are not required to have a useful transgenic animal.

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Other issued U. S. patents also confirm the routine practice of the teaching of the instant application for making and using APP-expressing transgenic animals. For example, U.S. Patent No. 5,877,399, issued to Hsiao *et al.* in 1999, discloses a transgenic mouse made according to the approach laid out in the instant application. Specifically, a cosmid vector comprising a DNA construct containing an APP sequence operably linked to promoter and enhancer sequences of, for example, the prion protein gene, was microinjected into fertilized mouse eggs for expression of APP in brain tissue. The patent states that "[a]ny amyloid precursor protein sequence can be used to produce the transgenic animals of the invention. (Emphasis added.) See col. 11, lines 46-47. The fertilized egg can then be implanted into a pseudo-pregnant female mouse until delivery. See col. 13, lines 40-52.

Another issued patent also confirms the routine practice of the teaching of the instant application for making and using APP-expressing transgenic animals. U.S. Patent No. 5,894,078, issued to Nalbantoglu *et al.* in 1999, discloses a transgenic mouse whose genome

comprises a nucleotide sequence encoding the carboxy-terminal 100 amino acids of human APP inserted into exon 1 of the neurofilament gene and under the control of the neurofilament regulatory region. This mouse exhibits increased amyloid deposition and other pathological signs of neurodegenerative disease, e.g., Alzheimer's Disease. The DNA construct was delivered by microinjection to the male pronuclei of fertilized mouse eggs which were then transplanted into pseudo-pregnant mice for full-term gestation. See col. 8, lines 23-34. This patent thus discloses that an APP nucleotide sequence or fragment thereof, when operably linked to one of several promoters, can be delivered to a fertilized egg and incubated in a pseudo-pregnant mouse for full term gestation and that these mice express the transgene.

A further example of recent art also confirms the routine practice of the teaching of the instant application for making and using APP-expressing transgenic animals. Li *et al.*, *Chinese Medical Journal*, 2001:114 (10), 1060-1063 (attached as Exhibit B) describes a transgenic animal that expresses human APP 770 and exhibits extracellular β-amyloid deposits in both the cortical and hippocampal brain regions. See page 1060, col. 2, paragraph 1.

The '399 patent, the '078 patent and Li *et al.* confirm that a person of skill, using the teaching of the instant application, can make transgenic mice that evidence a neuropathology associated with Alzheimer's Disease. Thus, these patents demonstrate that the Examiner's allegations of lack of enablement of the instant application are improper. In fact, the instant

specification provides substantial guidance when it discloses several constructs for producing a transgenic mouse. A person of skill, using the teachings of the pending application at the time of filing, could make a non-human transgenic animal, for example a mouse that expresses a mutant APP gene, without undue experimentation. Moreover, persons of skill in the art of transgenic non-human animals expect and consider reasonable the type and amount of experimentation involved in applying the teaching of the application to make such an animal. Thus, the claimed invention does not require undue experimentation.

The claimed invention is directed to transgenic animals that comprise and express an APP or a region of APP having the Swedish mutation. Prior to the present invention, several isoforms of APP were known, for example, APP 695, APP 714, APP 751 and APP 770, and these isoforms are described in the application as essentially interchangeable for the purpose of making the present transgenic animal. In contrast, prior to the present invention, the Swedish mutation was not known. Thus, a person of skill would not have expected or been able to take advantage of the fact that the Swedish mutation produces high levels of β-amyloid. The Swedish mutation produces more β-amyloid than other mutations, regardless of isoform, because it causes more cutting at the β-secretase site because the site is more attractive to the enzyme. It is this effect on the level of β-amyloid that has made transgenic animals that express this mutation particularly valuable as Alzheimer's Disease models, regardless of isoform. See, for example Hsiao '399, which describes the effect of APP 695 with the Swedish mutation on β-amyloid levels. The

claimed invention is directed to the Swedish mutation, characterized by the presence of a mutation at codon 670 and/or codon 671 which can be found in any of the known isoforms of APP that have the corresponding codons, for example, APP 695, APP 714, APP 751 and APP 770. Thus, the invention as described and claimed includes any form of APP having the Swedish mutation.

Nevertheless, the Office Action states that for a transgenic non-human animal, it was well established at the time of filing that not all transgenes will be expressed although they may be integrated into the genome. The Office Action, citing Kappell *et al.*, Current Opinion in Biotechnology 3, page 549, col. 2, paragraph 2 (1992), further states that transgenic animals have within their cells mechanisms that prevent expression of the transgene, e.g., DNA methylation or deletion from the genome.

The Examiner has incorrectly summarized the teachings of Kappell *et al.* What Kappell *et al.* actually states is that "[w]hile the investigator has the ability to target transgene expression to a large extent, there are inherent cellular mechanisms that may alter the pattern of gene expression. For example, DNA imprinting, resulting from differential CpG methylation, may affect transgene expression, depending on the sex of the parent from which the gene was inherited. Alternatively, a detrimental transgene may undergo somatic deletion." (Emphases added) Page 549, col. 2, paragraph 2. The Examiner improperly asserts that this reference teaches that cellular mechanisms are present that prevent expression of the transgene. In fact, the

reference teaches only that the cellular mechanisms may affect expression of the transgene, but it does not state that in all or even most cases that would happen. In fact, Kappell *et al.* is as supportive of the enablement of the claimed animals as it is a challenge to it. For example, Kappell *et al.* concludes that "[t]ransgenic animal technology is now well established as a critical method for analyzing gene expression and function. The approach continues to evolve, however, to include new strategies that offer a broad array of regulatory regimes." Page 551, col. 1, paragraph 1. Thus, in teaching that transgenic technology is well established, Kappell *et al.* shows that the making of transgenic non-human animals is routine. Besides, Kappell et al. does not specifically refer to APP, and the multiplicity of patents that have issued confirming Mullan '169 with examples of APP transgenics. These references prove that Kappell et al, if read to imply any problems with an APP- expressing transgenic animal, is wrong in any case.

The Office Action cites Lannfelt *et al.*, *Behav. Res.* 57, page 210, col. 1, paragraph 5 and col. 2, paragraph 4, lines 8-16 (1993) to allege that the production of transgenic mice expressing an APP transgene had been problematic and that the reason was mice as a species may be resistant to the formation of Alzheimer's related pathologies and that sufficient expression of the APP transgene may be difficult to achieve.

The Examiner has failed to take the teaching of Lannfelt *et al.* as a whole, so that the conclusion drawn is a negative overstatement of Lannfelt *et al.*'s teaching. Thus, it is error for the Examiner to rely on Lannfelt *et al.* to assert that the claimed invention is not enabled. In fact, Lannfelt *et al.* supports the utility and enablement of the presently claimed animals by suggesting that the approach applicant teaches is more likely than other approaches to produce a useful

animal. More specifically, Lannfelt *et al.* states that "... there are no published reports of the use of mutant AD pathogenic sequences in transgenics. It is likely that such sequences are more likely to lead to β-amyloid deposition. Unfortunately, this lack of reports almost certainly reflects the fact that most of the groups which have made such mice are either in commercial organizations, or sponsored by them." [Emphasis added.] See page 211, column 1, first full paragraph. Thus, rather than being relevant for teaching that the production of the present mutant APP mouse model would be problematic, Lannfelt *et al.* is highly relevant in its suggestions that mutant APP-expressing mice as claimed are more likely to be useful and that some of these transgenic mice had already been made.

The Office Action cites Higgins *et al.*, *Annals NY Acad. Sci.* (1993), and alleges that transgenic mice that express human APP 695 were known not to form amyloid protein deposits or neuritic processes and further alleges that making a transgenic mouse is problematic because mice as a species may be resistant to the formation of Alzheimer's pathologies and that sufficient expression of the APP transgene may be difficult to achieve.

The Examiner's assertion regarding the teaching of Higgins *et al.* is not supported in that reference. In fact, Higgins *et al.* teaches that, rarely, mice can express the human APP 695 gene and produce β-amyloid peptide. Moreover, what the Examiner fails to state is that Higgins *et al.* shows that transgenic mice that express the human APP 751 gene did indeed manifest neuropathologies associated with Alzheimer's Disease. Specifically, mice expressing the human APP 751 gene had preamyloid deposits that stained with a monoclonal antibody (mAb 4.1). (See page 225.) Further, the APP 751 mice showed evidence of numerous neuropil threads and

neurofibrillary tangles, detected with monoclonal antibody Alz50. These features are described as "features of early Alzheimer's disease (AD) pathology." See Abstract. Thus, rather than suggesting problems with the method taught by applicant, Higgins *et al.* supports success without undue experimentation.

It is important to note the expected similarity in expression of APP 751 and APP 770. Lannfelt *et al.* points out that APP 751 and APP 770 both contain a protease inhibitor domain which is important in amyloidogenesis. See page 211, column 1, first paragraph. Moreover, Quon *et al.* teaches that APP 695 lacks the Kunitz serine protease inhibitor that is found in APP 751 and APP 770 and that the presence of this protease inhibitor may result in an increase in mRNA encoding the APP, resulting in β-amyloid deposits in the brain. The absence of the Kunitz serine protease inhibitor in APP 695 may be a reasonable hypothesis; however, the hypothesis is wrong. Hsiao '399 shows use of the '695 isoform in producing transgenic mice that demonstrate Alzheimer's pathology, and the resultant strain, Tg2576, is the dominant model in commercial use today. Thus, the Examiner's allegation that mice as a species are resistant to expressing a mutated human APP gene is not supported. In fact, taking the success with APP 751 described by Higgins *et al.* and Quon *et al.* with the suggestion in Lannfelt *et al.* to use a mutant form of APP shows that those in the art would have expected success using the claimed mutant APP of the present invention and that making such transgenic non-human animal would be routine.

The Office Action, citing Felsenstein *et al.*, Alzheimer's and Parkinson's Diseases, I. Hanin ed., Plenum Press, New York (1995), page 406, page 1, goes on to state that at the time of filing

of the pending application, the art taught that transgenic rats containing an APP transgene failed to demonstrate any Alzheimer's related pathology at six months of age.

Felsenstein *et al.* may be technically correct, but misses a key point of the art, when it states that "[t]o date no animal model exists that can recapitulate the pathological cascade of AD." (See page 401, paragraph 1, last sentence.) The fact is that Quon *et al.* described a credible mouse model for studying a neuropathology of Alzheimer's Disease four years before the publication of Felsenstein *et al.*, and Higgins *et al.* was published two years before the publication of Felsenstein *et al.* (See above.) Furthermore, it is recognized in the art and by the Office (See, e.g., the '936 patent) that a useful model may allow the study of APP processing (in AD or Down's Syndrome) without necessarily recapitulating the pathological cascade of AD.

Further, it should be noted that Felsenstein *et al.* recognizes that rats may not be the proper model for studying Alzheimer's Disease, stating "[t]ransgenic work with the fusion protein will continue with the introduction of the early onset FAD mutations as well as looking into alternative species which may be more prone to initiation of amyloidosis" (page 407, last paragraph, last sentence) (Emphasis added). Thus, the fact that a rat model failed to show characteristic neuropathological signs of Alzheimer's Disease is not particularly relevant to the likelihood of success in other rodents or other non-human animals. The instant application provides a credible mouse model, and the art, at the time of filing of the application, showed that a mouse model of Alzheimer's Disease did produce neuropathological signs of the disease (See, e.g., Quon *et al.*).

Furthermore, in Felsenstein *et al.*, the transgene encoded a fusion protein which comprised an APP and a reporter protein. Although the encoded fusion protein was expressed in rats, no amyloid deposits were found on histopathological examination of rats six months old. The reference admits that older rats with the transgene, in which it would be more likely to find amyloid deposits, were not examined and that the proteolytic processing of the fusion protein in rats might not be the same as was found in *in vitro* studies (page 407, last paragraph). In contrast, in Higgins *et al.*, the APP 751 gene was not expressed as a fusion protein, and amyloid was found in brain tissue of mice. Higgins *et al.* also points out that in mice, older animals at the end of their natural life span had twice the frequency of amyloid deposits than younger animals. See Higgins *et al.*, Table 2, page 226. Thus, because the fusion of a reporter protein to the mutated APP may have interfered with the deposition of amyloid, and because older rats were not examined, Felsenstein *et al.* is not relevant art.

Based on the successes in the art and the teachings of the instant application, persons of skill in the art, having the knowledge of the art, could make mice that express mutant APP 770 transgene and have amyloid deposits in their brain. Furthermore, the more recent art includes several examples of transgenic animals made in the manner as disclosed in the present application. Thus, the instant application teaches how to make and use a transgenic non-human animal that expresses a specific mutation of human APP 770 wherein the amino acid at 670 is an amino acid other than lysine and/or the amino acid at 671 is an amino acid other than methionine. Applicant believes that Quon *et al.* and the '742 patent are evidence that before the filing date of the instant application, making transgenic mice that express an APP 770 gene was routine and did not require undue experimentation. Moreover, Li *et al.* and the '936, '078, and '399 patents

confirm that the teaching of the instant application is enabled. Thus, applicant believes that the rejection of claims 21-32 based on lack of enablement is not supported and respectfully requests that this rejection be withdrawn.

The present rejection appears to be based primarily on a utility/enablement component because it unquestionably calls for evidence of efficacy, i.e., utility. Applicant notes that regardless of the statutory basis for the rejection, applicant is not required under section 112, first paragraph (or section 101) to demonstrate that the claimed transgenic mice have β-amyloid deposits in their brain. At most, applicant is required to provide evidence of likely success that would be believed by a person of skill, i.e., credible evidence. Applicant respectfully asserts that the specification read in light of the art supports the claimed use with evidence that rises to at least and above the level of credibility. If the Office cannot show that applicant's assertions regarding how to use the claimed inventions are objectively incredible, this rejection should be withdrawn. In fact, the Office does not give any specific scientific basis (i.e., specific to the claimed composition) that contradicts applicant's explicit and reasonable teaching regarding how to use the claimed transgenic mice. In the absence of such a scientific basis, the Office should accept applicant's teaching and find enablement.

The Office Action notes that in claims 21 and 23 it is stated that the nucleic acid is contained within the germ or somatic cells. The Office Action acknowledges that the specification teaches the production of transgenic non-human animals where the nucleic acid sequence is integrated into the genome and passes through the germ line to progeny; however, the Office Action alleges that claims 21 and 23 are not enabled because the specification does not

enable chimeric non-human mammals where the nucleic acid is episomal or only in somatic cells. The Office Action goes on to state that claims 21 and 23 need to state that the nucleic acid is operably linked to a promoter as this is the only means of expressing enabled by the specification.

Applicant teaches how to make a transgenic non-human animal with the nucleic acid contained only in somatic cells. The instant specification discloses using viral vectors to deliver the mutated gene to a developed or developing animal. Delivering the nucleic acid to a developed or developing animal would put the transgene into the somatic cells of the animal. See in the specification page 22, lines 15-18. Thus, claims 21 and 23 are enabled.

Applicant teaches how to make a chimeric non-human animal. A chimeric animal is an animal in which at least one but not all of the cells of its body comprise and express the exogenous nucleic acid. The specification teaches microinjection of an embryonic stem cell containing the mutated transgene into a host blastocyst and subsequent incubation of the embryo in a foster mother. Because not all of the cells in the blastocyst contain the transgene, the developing embryo by definition is chimeric. See in the specification page 22, lines 1-6. Thus, claims 21 and 23 are enabled.

The Office Action further states that as for isolated cells, the claims do not provide for an assay as there is allegedly no statement that the nucleic acid is operably linked to a promoter or that the APP 770 is produced to detectable levels. The Office Action states that the specification

only enables expression of a nucleic acid sequence by a promoter and expression to a detectable level.

Claims 21 and 23 are amended to include the phrase "operably linked to a promoter." Support can be found in the specification on page 13, lines 1-9. Adding the phrase "operably linked to a promoter," as suggested by the Examiner, overcomes these rejections.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 25-32 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. Specifically, the Office Action states that the term "immortalized cell line" in claims 25-28 is confusing because only mammalian cells are considered "immortal." The Office Action goes on to state that the subject matter of "immortalized cell line" is not clear.

Claim 25 is amended herein to recite "[a]n isolated cell containing a nucleic acid characteristic of human amyloid precursor protein including the nucleotides encoding codon 670 and 671 of human amyloid precursor protein 770, operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and, wherein the cell expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671." Claim 26 is amended herein to recite "[t]he cell of claim 25, wherein the cell is immortalized." Applicant believes that the amendment of claim 25, deleting the term "host" and substituting therefor the term "an isolated cell," and the amendment of claim 26 deleting the term "host" and substituting therefor the term "the cell," overcome these

rejections and indicate that the cell is immortalized. Further, claim 26 is amended herein to correct an inadvertent typographical error; specifically, claim 26 is amended to depend from claim 25 instead of claim 23. Therefore, applicant respectfully requests that these rejections be withdrawn and that amended claims 25 and 26 be allowed.

Further, claim 27 is amended herein to recite "[a]n isolated cell containing a nucleic acid characteristic of human amyloid precursor protein including the nucleotides encoding codon 670, 671 and 717 of human amyloid precursor protein 770, operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717 and, the cell expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717." Claim 28 is amended herein to recite "[t]he cell of claim 27, wherein the cell is immortalized." Applicant believes that the amendment of claim 27, deleting the term "host" and substituting therefor the term "an isolated cell," and the amendment of claim 28, deleting the term "host" and substituting therefor the term "the cell," overcome these rejections and indicate that the cell is immortalized. Therefore, applicant respectfully requests that these rejections be withdrawn and that amended claims 27 and 28 be allowed.

The Office Action goes on to state that the term "capable of" in claims 29-32 is not clear because the specification does not disclose those factors that make the agents capable of or not capable of treating Alzheimer's Disease.

Claims 29-32 are amended herein, deleting the term "capable of" and adding before "treating" the term "for." Deleting the term "capable of" from claims 29-32 overcomes these rejections. Support can be found in the specification on page 20, lines 18-20. Therefore, applicant respectfully requests that these rejections be withdrawn and that amended claims 29-32 be allowed.

The Office Action states that the word "promotes" in claims 29 and 30 does not clearly indicate the relationship between expression of the transgene and the development of Alzheimer's Disease neurological characteristics. The Office Action goes on to state that the phrase "results in" would be clearer.

Applicant respectfully points out that the word "promotes" does not appear in claims 29 and 30, but does appear in claims 22 and 24. Claims 22 and 24 are amended herein, according to the Examiner's suggestion, deleting the word "promotes" and substituting therefor the phrase "results in." Applicant believes that these rejections are overcome and respectfully request that these rejections be withdrawn and that amended claims 22 and 24 be allowed.

A Credit Card Payment Form PTO-2038 authorizing payment in the amount of $640.00 ($180.00 representing the fee under 37 C.F.R. § 1.17(p) and $460.00 for a three-month extension of time fee) is enclosed. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents, Washington, D.C. 20231, on the date shown below.

Gwendolyn D. Spratt

Gwendolyn D. Spratt

12-13-02

Date

VERSION WITH MARKINGS TO SHOW CHANGES MADE

21. (Amended) A transgenic non-human animal [containing] comprising, in a germ or somatic cell, [the] a nucleic acid [of claim 1] characteristic of human amyloid precursor protein including the nucleotides encoding codon 670 and 671 of human amyloid precursor protein 770, operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and, wherein the animal expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671.

22. (Amended) The animal of claim 21, wherein expression of the nucleic acid [promotes] results in neuropathological characteristics of Alzheimer's disease in the animal.

23. (Amended) A transgenic non-human animal [containing] comprising, in a germ or somatic cell, [the] a nucleic acid [of claim 9] characteristic of human amyloid precursor protein including the nucleotides encoding codon 670, 671 and 717 of human amyloid precursor protein 770, operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717 and, wherein the animal expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717.

24. (Amended) The animal of claim 23, wherein expression of the nucleic acid [promotes] results in neuropathological characteristics of Alzheimer's disease in the animal.

25. (Amended) [A host] An isolated cell [containing the] comprising a nucleic acid [of claim 1] characteristic of human amyloid precursor protein including the nucleotides encoding codon 670 and 671 of human amyloid precursor protein 770 operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670

and/or an amino acid other than methionine at codon 671 and, [which host] wherein the cell expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671.

26. (Amended) [A host] The cell of claim 2[3]5, wherein the [host] cell is [an] immortalized [cell line].

27. (Amended) [A host] An isolated cell [containing the] comprising a nucleic acid [of claim 9] characteristic of human amyloid precursor protein including the nucleotides encoding codon 670, 671 and 717 of human amyloid precursor protein 770, operably linked to a promoter, wherein the nucleic acid encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717 and, [which host] the cell expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671 and an amino acid other than valine at codon 717.

28. (Amended) The [host] cell of claim 27, wherein the [host] cell is [an] immortalized [cell line].

29. (Amended) A method of screening for an agent [capable of] for treating Alzheimer's disease comprising contacting the animal of claim 22 with the agent and monitoring the expression, processing or deposition of amyloid precursor protein or fragments thereof.

30. (Amended) A method of screening for an agent [capable of] for treating Alzheimer's disease comprising contacting the animal of claim 24 with the agent and monitoring the expression, processing or deposition of amyloid precursor protein or fragments thereof.

31. (Amended) A method of screening for an agent [capable of] for treating Alzheimer's

disease comprising contacting the [host] cell of claim 25 with the agent and monitoring the expression or processing of amyloid precursor protein or fragments thereof.

32. (Amended) A method of screening for an agent [capable of] for treating Alzheimer's disease comprising contacting the [host] cell of claim 27 with the agent and monitoring the expression or processing of amyloid precursor protein or fragments thereof.

Formation of β-amyloid protein deposits in brains of transg nic mice

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DEPOSITS of β-amyloid are one of the main pathological characteristics of Alzheimer's disease. The β-amyloid peptide constituent (relative molecular mass 4,200) of the deposits is derived from the β-amyloid precursor protein (β-APP) which is expressed in several different isoforms[1–6]. The two most prevalent β-APP isoforms are distinguished by either the presence (β-APP751) or absence (β-APP695) of a Kunitz serine protease inhibitor domain. Changes in the abundance of different β-APP messenger RNAs in brains of Alzheimer's disease victims have been widely reported[7–12]. Although these results have been controversial, most evidence favours an increase in the mRNAs encoding protease inhibitor-containing isoforms of β-APP and it is proposed that this change contributes to β-amyloid formation[9–12]. We have now produced an imbalance in the normal neuronal ratio of β-APP isoforms by preparing transgenic mice expressing additional β-APP751 under the control of a neural-specific promoter. The cortical and hippocampal brain regions of the transgenic mice display extracellular β-amyloid immunoreactive deposits varying in size (≤5–50 μm) and abundance. These results suggest that one mechanism of β-amyloid formation may involve a disruption of the normal ratio of neuronal β-APP isoform expression and support a direct relationship between increased expression of Kunitz inhibitor-bearing β-APP isoforms and β-amyloid deposition.

A chimaeric gene was constructed between the human β-APP751 complementary DNA and the rat neural-specific enolase (NSE) promoter, termed NSE: β-APP751. The rat NSE promoter directs the neural-specific expression of β-galactosidase in transgenic mice[13] and we have confirmed this using a NSE promoter fragment slightly truncated at the 5′ terminus (our unpublished results). The promoter fragment containing the 5′ untranslated region of NSE and a roughly 1.2-kilobase (kb) intron in this domain was fused to the β-APP751 cDNA such that the initiator methionine of NSE was replaced with the initiator methionine of β-APP751. Nine of 44 mice that developed from embryos injected with NSE:β-APP751 DNA carried the transgene. Three pedigrees were selected for extensive characterization: founders 10 (F10), 11 (F11) and 24 (F24). Homo- and hemizygotic states and transgene copy numbers were determined by comparison to the endogenous single copy β-APP mouse gene using Southern blot hybridization with a probe common to both mouse β-APP and human β-APP751 (Table 1).

RNA expression of the inherited transgenes in the three pedigrees was investigated. Total brain RNA was isolated both from positive and from wild-type control animals, reverse transcribed, and a specific DNA subfragment amplified by polymerase chain reaction (PCR). Primers for PCR were designed such that only transcripts derived from the transgene would be amplified, that is, one primer hybridizes to the NSE 5′ untranslated region and the other to the 5′ coding domain of β-APP. The NSE PCR primer corresponds to a site located upstream of the intron so that amplification of contaminating genomic DNA or unprocessed transcripts could be detected. A predicted 373-base pair (bp) fragment is amplified from reverse-transcribed RNA prepared from each transgenic animal but not from wild-type mice (Fig. 1*a*). As a control, half of the reverse-transcribed RNA was amplified with a primer for the native

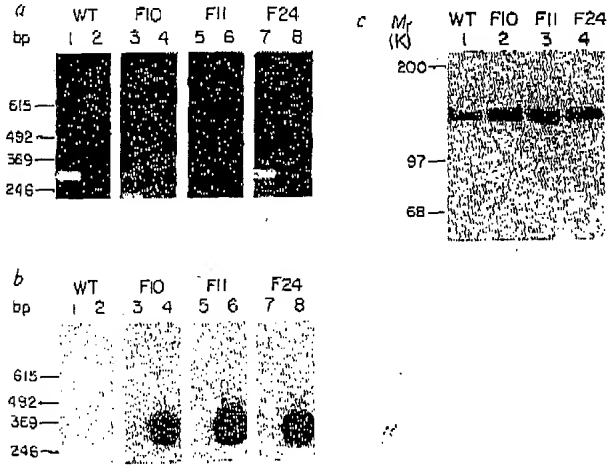

FIG. 1 NSE: β-APP751 expression in brain. *a*, Agarose gel electrophoresis of reverse transcribed PCR products visualized with ethidium bromide staining. Even numbered lanes, reverse transcribed PCR products from wild-type (WT), NSE: β-APP751 founder 10 (F10), founder 11 (F11) and founder 24 (F24) RNA primed with primers specific for NSE: β-APP751 RNA. Odd numbered lanes, reverse-transcribed PCR products from RNA primed with primers specific for endogenous β-APP RNA. *b*, Southern blot of above reverse-transcribed PCR products using ^{32}P-labelled oligonucleotide probe specific for the NSE: β-APP751 chimaeric gene. Lanes are the same as in *a*. *c*, Western blot analysis of β-APP in brain. Lane 1, WT; 2, F10; 3, F11; 4, F24 total brain protein immunoblotted with β-APP antiserum.

METHODS. An ~8-kb rat genomic fragment containing the NSE gene was isolated on the basis of the published sequence[22] and the 2.3-kb fragment used for the chimaeric gene isolated by PCR. PCR primers were designed to generate *Bgl*II and *Nco*I sites at the 5′ and 3′ terminus of the fragment, respectively. A *Nru*I(position 123)–*Xmn*I(position 2,665) fragment was isolated from β-APP751 cDNA[4], and was ligated with the 2.3-kb NSE fragment harboured in a derivative of pcDV1 plasmid containing the simian virus 40 (SV40) late region polyadenylation signal[23]. A linear fragment of NSE: β-APP751 was prepared by cleavage with *Sal*I and *Nde*I and was injected into fertilized embryos of the JU strain of mouse[24]. The JU strain, developed by Eric Bradford at the University of California Davis, was chosen for its large litter size. For genotype and copy number determinations, 40 μg of tail DNA was digested with *Bgl*II and electrophoresed on 0.8% agarose gels. Southern blots were prepared[25] and hybridized with an oligonucleotide probe (5′-ATGGATGTGTACTGTTTCTTCTTCA-3′) radiolabelled with ^{32}P by T4 kinase. Blots were hybridized at 60 °C in 6 × SET (1 × SET = 0.15 M NaCl, 30 mM Tris-HCl pH 8.0, 2 mM EDTA) with 5 × Denhardt's solution and washed at 60 °C for 40 min in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate). For transcriptional analyses, 2 μg of total brain RNA was reverse transcribed with oligo(dT)$_{12-18}$, after which the reaction was divided into two equal aliquots. PCR[26] was done on one aliquot using NSE (5′-CACCGCCACCGGCTG-AGTCTGCAGTCCTCG-3′) and β-APP (5′-TCTTGCACTGCTTGCGGCCCCGCTT-GCACC-3′) primers and on the second aliquot with the same β-APP primer and a primer to the secretory signal sequence of β-APP (5′-TTGGCACTGCT-CCTGCTGGCCGCCTGGACG-3′) in place of the NSE primer. DNA products were electrophoresed on 2% agarose gels, visualized by staining with ethidium bromide, then Southern blotted[25] and hybridized with a ^{32}P-labelled oligonucleotide probe to the NSE: β-APP751 fusion sequence (5′-AGATCCCAGC-CACCGATGCTGCCCGGTTTG-3′). Blots were hybridized at 65 °C in 6 × SET and washed at 65 °C for 40 min in 4 × SSC. Protein homogenates were made from total brain[14] and 50 μg of each sample was electrophoresed on 7.5% SDS–polyacrylamide gels[27]. A western blot[28] was developed using a 1:500 dilution of antiserum and ^{125}I-labelled protein A. The antiserum was raised against full-length human β-APP695 expressed by a recombinant vaccinia virus[18]. Identically prepared gels stained with Coomassie blue dye confirmed that equivalent amounts of protein were loaded for each sample.

β-APP secretory signal sequence and the same 5′ coding domain β-APP primer to produce a 307-bp DNA fragment representing amplification from endogenous β-APP RNA (Fig. 1*a*). When all the PCR reaction products are hybridized with a probe bridging the junction between NSE and β-APP751 sequences, only the products derived from the transgenic brains hybridize, documenting the authenticity of the 373-bp PCR product (Fig. 1*b*).

Western blots were made to evaluate changes in protein expression in the brains of the transgenic animals. Equal amounts of total protein from whole brain homogenates were electrophoresed on polyacrylamide gels, transferred to a membrane then reacted with polyclonal serum raised against full-length β-APP. A band (or set of unresolved bands) of relative molecular mass of about 130,000 (130K), corresponding to the reported average size of mammalian brain β-APP isoforms[14–16], is observed in the control, as well as in each transgenic protein homogenate (Fig. 1*c*). This signal is increased in the NSE:β-APP751 samples relative to the wild-type sample suggesting globally elevated β-APP751 expression in the transgenic brains. To obtain a more refined examination of NSE:β-APP751 expression and its effects, we used immunocytochemistry.

A panel of monoclonal antibodies was prepared using a synthetic peptide corresponding to residues 1–28 of the β-amyloid protein as the immunogen. The specificity of the monoclonals was established by immunoperoxidase staining of brain sections from Alzheimer's disease victims (Fig. 2 *a*, *b*). Sections were prepared from brains of NSE:β-APP751 transgenic mice, as well as from wild-type mice and both were stained in parallel with one monoclonal, 4.1 (Table 1). Reproducibly greater immunoperoxidase reactivity is observed in neurons and as fine puncta throughout the neuropil of the transgenic brains compared with the immunoreactivity visualized in brains from wild-type mice. A pronounced staining of neuritic processes is also noticeable (Fig. 2*d*). This enhancement of arbor-forming neuronal processes is most evident in the stratum flanking the pyramidal cell layer of the CA-1 and CA-3 regions of transgenic hippocampi. Both neuronal and process staining are fully competed by earlier incubation of the antibody with the synthetic β-amyloid peptide. Neuritic staining in the transgenic brains is also detected using antibodies raised against full-length β-APP (Fig. 2*c*), indicating full-length β-APP is present in the neuritic

TABLE 1 Summary of mice used for immunohistology

Line	Animal	Sex	Age*	Genotype	Copy number†	Deposits‡
NSE:β-APP751						
10	0	F	12	Aa	1	+++
	31§	F	7	Aa		++
	168	F	5	Aa		+++
	334	M	2	AA		+
11	0	M	15	Aa	4	+++
	51	M	12	Aa		+
	236	M	4	AA		+++
	287	F	3	AA		+
24	77	M	8	Aa	8	+
	201	F	5	AA		+
Wild type						
	1	F	4	NA	NA	—
	2	F	4			—
	3	M	5			—
	4	M	3			—
	5	M	9			—
	6	F	12			—
	7	M	14			—

M and F indicate male and female mice, respectively; AA and Aa represent homozygotic and hemizygotic animals, respectively; NA, not applicable.
* Months.
† Haploid.
‡ >5 μm in size, (–) none, (+/–+/+++) relative abundance of deposits, that is, + indicates <5; ++ indicates 5–10; +++ indicates >10 deposits per section as an average of multiple sections stained.
§ NSE: β-APP751 F10, number 31 died of unknown cause.

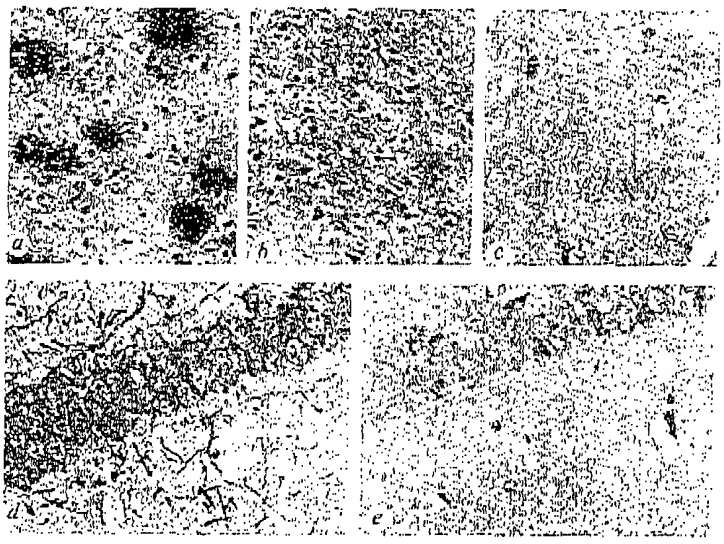

FIG. 2 Immunoperoxidase staining of human and mouse brain. Human Alzheimer's disease tissue section from caudal hippocampus stained with 4.1 antibody without (*a*) and an adjacent section (*b*) with preincubation with the β-amyloid synthetic peptide immunogen (×250). Hippocampal CA-1 field of NSE: β-APP751 F10 (number 334) stained with antibodies to full-length β-APP (*c*); pyramidal cell layer of CA-1 region from NSE: β-APP751 F10 (number 334) stained with 4.1 antibody (*d*); same region from wild-type mouse (number 3) identically stained with 4.1 antibody (*e*) (×500).

METHODS. A synthetic peptide corresponding to residues 1–28 of the β-amyloid protein[1,2] was prepared and self-aggregated by freezing and thawing. The peptide aggregate was mixed with methylated bovine serum albumin and adjuvant for immunizing and boosting mice. Hybridomas from the sensitized spleen cells were generated[29]. Clones secreting anti-peptide antibodies were expanded and subcloned by limiting dilution. The epitope recognized by each monoclonal was mapped to the N-terminal 10 residues of the β-amyloid protein. For analysis of mouse brains, brains were removed and fixed with 4% paraformaldehyde, embedded in paraffin and 6 μm coronal midbrain sections made. Sections were deparaffinized, rehydrated, treated for 30 min with 0.3% H_2O_2, then with 80% formic acid for ~2 min. Sections were next incubated at 37°C for 30 min with a 1/20 dilution of conditioned medium from the hybridoma secreting the 4.1 antibody. An anti-mouse avidin-biotinylated horseradish peroxidase (ABC) kit was used according to supplier's recommendations (Vector, Burlingame, California) and the horseradish peroxidase visualized with 3,3′-diaminobenzidine. Staining for full-length β-APP used the antiserum used for western blotting in *c* at a 1:500 dilution and an anti-rabbit ABC kit. Formic acid treatment was omitted for β-APP staining. Sections were counterstained with haematoxylin and eosin. Human brain sections were from individuals clinically diagnosed with Alzheimer's disease. The human sections were prepared and stained identically as mouse tissue sections except they were treated with 98% formic acid for 10 min. For competition experiments, the antibody diluent was preincubated at 4 °C for 12 h then 37 °C for 30 min with 250 μg ml^{-1} 1–28 β-amyloid synthetic peptide before application. Congo red, thioflavin S and silver staining were done using published procedures[18–21,30].

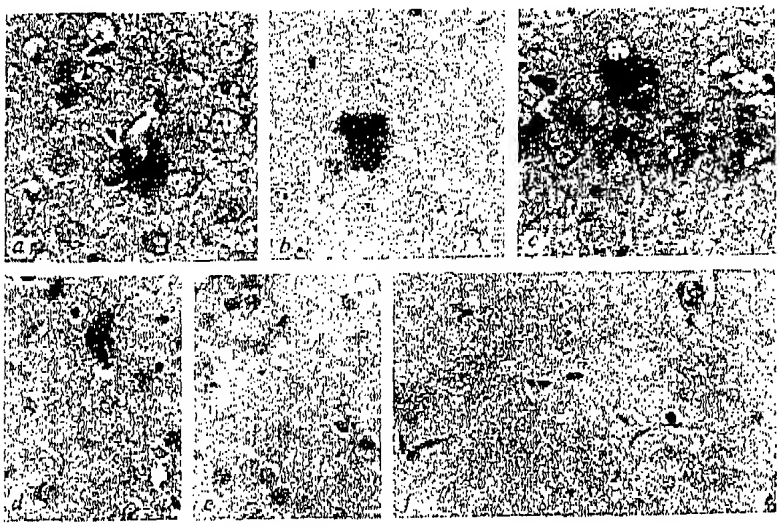

FIG. 3 Immunoreactive deposits in NSE:β-APP751 brains. a, Compact deposit in frontalparietal cortex of F11 (number 0); b, compact deposit in thalamus of F11 (number 236); c, compact deposit in hippocampal CA-2 field of F11 (number 0); d, cluster of deposits in frontalparietal cortex of F10 (number 168); e, adjacent section as in d but antibody preincubated with β-amyloid synthetic peptide before staining (arrowheads demark same capillaries in field of d and e); f, amorphous deposits in the hippocampal stratum moleculare of F11 (number 236) (×500). Immunocytochemistry and competition were performed as described in the legend to Fig. 2.

processes, although the possibility that the neurites also contain β-amyloid protein cannot be excluded.

Extracellular immunoreactive deposits are also consistently seen in the brain sections from each of the three transgenic lines stained with the 4.1 monoclonal which are not seen in sections from wild-type animals identically stained. The immunoreactive deposits vary in size, shape and frequency. Examples of compact deposits 10–50 μm in diameter are shown in Fig. 3*a–d*. These immunoreactive deposits tend to occur in clusters and are most frequently observed in the cortex and hippocampus although occasionally they have been found in the thalamus and striatum. A second type of immunoreactive extracellular deposit is reproducibly seen in transgenic brain sections which is lacking in control brain sections. This type of deposit is diffuse, amorphous and granular (Fig. 3*f*). Detection of extracellular deposits in the tissue sections from the transgenic animals required treatment with formic acid. Immunoreactivity of these structures also could be competed by the β-amyloid peptide (Fig. 3*e*). In a preliminary survey, antibodies to full-length β-APP stained extracellular deposits of similar morphology as those stained by the 4.1 monoclonal. Also, in general, the deposits are stained by silver salts, infrequently by thioflavin S, but not by Congo red, corroborating a preamyloid-like composition (data not shown). Owing to the small group of animals analysed, it is difficult to make a correlation between the frequency of deposit appearance with age, genotype or sex (Table 1).

Although there may exist several different mechanisms promoting β-amyloid formation[17], the observed increased level of Kunitz inhibitor-containing β-APP isoform RNA in neurons of Alzheimer's disease brains suggests that Kunitz inhibitor β-APP isoform overexpression may be one mechanism[9–12]. The three NSE:β-APP751 transgenic lines which have moderately increased neuronal expression of β-APP751 and form extracellular β-amyloid immunoreactive deposits (as well as our preliminary data on NSE:β-APP695 transgenic lines which do not) support this hypothesis. The two types of extracellular deposits, diffuse and compact, seen in the transgenic mice resemble several β-amyloid structures typically seen in the brains of Alzheimer's disease victims, specifically preamyloid and preamyloid plaques[18–21]. It will be of interest to determine whether the quality and/or quantity of deposits change in an age-dependent manner and if the mice display other pathological features characteristic of Alzheimer's disease. □

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Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores

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Inositol 1,4,5-trisphosphate ($InsP_3$) functions as a second messenger to mobilize Ca^{2+} from intracellular reservoirs[1]. The release mechanism displays all-or-none characteristics[2,3], that may account for other observations that the $InsP_3$-induced mobilization of Ca^{2+} is quantal[4–6]. Quantal release may depend on the sensitivity of the $InsP_3$ receptor being regulated by the Ca^{2+} concentration in the lumen of the endoplasmic reticulum[7]. We report here that

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Spatial learning deficits in amyloid precursor protein 770 transgenic mice

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Keywords: *Alzheimer's disease · cholinergic system · learning · transgenic*

Objective To determine whether learning deficits could be seen in transgenic mice expressing human amyloid precursor protein 770 (APP_{770}).

Methods Female heterozygous transgenic and nontransgenic mice aged 3, 6 and 9 months at the start of testing were used, with eight mice in each age group. All mice were subjected to various behavioral tasks including the Y-maze task and the Morris water maze. After behavioral testing, the mice were sacrificed, and their brain tissues were used for measuring the choline acetyltransferase (ChAT) activity.

Results Nine-month-old transgenic mice exhibited spatial learning deficits in the Morris water maze and in spontaneous alternation in the Y-maze, compared with those of the age-matched non-transgenic mice. The behavioral changes accompanied a reduction of ChAT activity in the cortical and hippocampal regions of transgenic mice. On the other hand, these behavioral deficits were not observed in transgenic mice either at 3 or at 6 months of age, in which ChAT activity remained unchanged.

Conclusions The present results show that the learning impairment observed in 9-month-old APP_{770} transgenic mice are accompanied by a decrease in cortical and hippocampal ChAT activities. This suggests that cholinergic deficits may be involved in the learning impairment observed in these APP_{770} mice. This model will be a useful tool in advancing our understanding of the relationship between the cholinergic system and the cognitive deficits observed in Alzheimer's disease (AD).

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Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by a progressive deterioration of memory and learning.[1] Prominent histopathological features of this disease which include extracellular deposition of β-amyloid (Aβ), intracellular neurofibrillary tangles (NFTs) and neuronal degeneration,[2] occur mostly in the cerebral cortex and hippocampal regions which play major roles in memory and cognition.[3]

Although the relationship between molecular neuropathologic changes and cognitive disturbances remains obscure, there is extensive evidence suggesting that dysfunction of the central cholinergic system contributes to the cognitive impairment in AD. The decrease in the activities of cortical choline acetyltransferase (ChAT) in AD and its correlation with cognitive impairment is remarkably consistent.[4] Moreover, pharmacological disruption of the cholinergic system in normal humans and animals induces learning and memory deficits which mimic those observed in AD.[4] These data provide considerable support for the hypothesis of an important involvement of the cholinergic system in the cognitive deterioration of dementia.

The development of valid animal models would facilitate the efforts on testing the cholinergic hypothesis and developing pharmacological agents. We have established a transgenic mouse model using human amyloid precursor protein 770 (APP_{770}) cDNA. Immunoblot analysis revealed transgenic APP_{770} expression in the transgenic mouse brain at levels > 5-fold higher than endogenous mouse APP levels. These transgenic mice also exhibit robust extracellular Aβ deposits as well as a significant reduction in ChAT activities in both cortical and hippocampal brain regions.[5] Moreover, these transgenic mice show a selective loss of basal forebrain cholinergic neurons.[6] We therefore tested whether

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cognitive deficits could be seen in the APP_{770} transgenic mice and whether these deficits occur parall with the cholinergic changes.

METHODS

Animals

APP_{770} transgenic mice were bred from a transgenic line produced and described previously.[5] Female heterozygous transgenic and non-transgenic mice aged 3, 6 and 9 months at the start of testing were used, with eight mice in each age group. Non-transgenic mice served as controls, with eight mice in each age group. All mice were housed in a temperature-controlled environment (22 – 23℃) and kept on a 12 h light-12 h dark cycle.

Straight alley

A straight alley, 100 cm long by 11 cm wide with 45 cm high sides, was placed in a large water tank filled to a depth of 30 cm with opaque water. An 11 cm^2 platform was placed at one end of the alley and was hidden 1 cm below the surface of the water. Each mouse was placed at the start of the alley, and the time taken to swim along the length of the alley to escape onto the platform was recorded. Since the distance between the starting point and platform was fixed, the latency of response expressed as s/m gives a measure of swimming speed. Each animal was given six trials and allowed 30 seconds on the platform between each trial.

Plus-maze activity

The plus-maze was made of plywood and consisted of two open arms (23.5 cm × 8.0 cm) and two enclosed arms (23.5 cm × 8.0 cm × 20 cm). The plus-maze was elevated to a height of 50 cm above the floor. The open arms and the central platform were painted white, and the enclosed arms were painted black. Each mouse was tested by placing it in the center of the plus-maze. The number of entries into the open and enclosed arms, respectively, and the total time spent in the enclosed arms were recorded during a period of 6 minutes. An entry was scored when the mouse entered the arm with all four feet.

Spontaneous alternation in Y-maze

The Y-maze consisted of a symmetrical 3-armed maze made of gray Plexiglas, with each arm being 50 cm long and 10 cm wide with 40 cm walls, radiating at an angle of 120℃ from each other. Mice were placed in the maze and allowed to freely explore for 5 minutes. Arms were arbitrarily labeled A, B and C, and sequence of arm entries was used to measure alternation behavior. An unbiased alteration score and a measure of position bias were calculated.

Spatial learning

A water maze based on that described by Morris[7] was constructed for mice. It was a 90 cm-diameter black cylindrical pool filled with water. A 6 cm × 6 cm transparent platform was placed at a fixed location in the pool, 1 cm below the surface of the water. The platform either remained hidden or was rendered "visible" by attaching a cue to signal position of the platform. The pool was always maintained in the same position in the room. Each mouse received 3 trials per day for 6 consecutive days, with each trial from a different starting point at the perimeter of the pool. The time taken to swim to the hidden platform to escape from the water (within 100 seconds) was recorded. The mouse was allowed to remain on the platform for 30 seconds before commencing the next trial. At the end of 3 trials, the mouse was removed, dried and placed under a lamp to warm up. After place learning performance, all mice were given a probe trial in which they swam for 100 seconds in the water maze with the platform removed. The percentage of time spent in each quadrant and the number of times the mice crossed the exact former platform location (platform crossing) during probe trial were recorded.

ChAT assay

After behavioral testing, the mice were sacrificed, and the brain tissues were removed for ChAT assay. ChAT activity was measured as described previously.[5]

Statistical analysis

Data were presented as $\bar{x} \pm s$. ANOVA and the Student's t test were used to analyze the data. $P < 0.05$ was considered significant.

RESULTS

Straight alley test of swimming speed

Latencies to escape onto the platform decreased progressively with repeated testing for all age groups. No significant difference was found between the transgenic and non-transgenic mice at 3, 6 or 9 months of age.

Plus-maze test of anxiety

There was no significant difference in plus-maze performance between the transgenic and non-transgenic mice at 3, 6 or 9 months of age.

Spontaneous alternation

The results of the spontaneous alternation test showed that there was no significant difference in the percentage of alternation between the transgenic and non-transgenic mice at either 3 or 6 months of age. In the 9-month-old group,

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however, the percentage of alternation decreased significantly ($P < 0.05$) in the transgenic mice (59.0% ± 2.9%) relative to the age-matched non-transgenic mice (69.8% ± 1.6%). A similar result was obtained when alternation was corrected for possible turning bias, with the 9-month-old transgenic mice (59.1% ± 3.0%) again significantly different from the age-matched non-transgenic mice (68.7% ± 1.6%). All age groups showed a similar degree of position bias and level of total arm entries (data not shown).

Spatial learning

The performance of transgenic mice, aged 3 or 6 months, in place learning was not significantly different from that of age-matched non-transgenic mice. The 9-month-old transgenic mice, however, were impaired in their performance in place learning, showing significantly higher escape latency to the hidden platform on days 3, 4, 5 and 6 compared with age-matched non-transgenic mice (Fig.). All mice were also subjected to a probe trial. The percentage of time spent in the quadrant of maze that formerly contained the platform (target quadrant) and the mean target platform crossing were recorded for both transgenic and non-transgenic mice. Nine-month-old transgenic mice spent significantly less time ($P < 0.05$) in the target quadrant (28.2% ± 4.7%) relative to age-matched non-transgenic mice (51.1% ± 6.1%). The mean target platform crossing was also significantly less ($P < 0.05$) for 9-month-old transgenic mice (34.8% ± 3.6%) relative to age-matched non-transgenic mice (45.4% ± 4.2%). Such differences were not found between transgenic and age-matched non-transgenic mice at either 3 or 6 months of age.

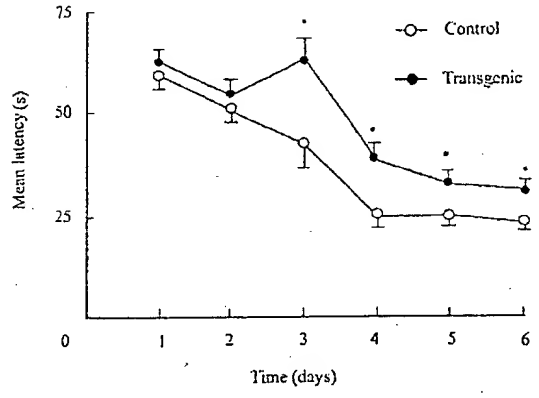

Fig. Water maze performances over 6 days in place learning of 9-month mice. * $P < 0.05$, compared with age-matched non-transgenic mice.

To rule out the possibility that the poorer performance of 9-month-old transgenic mice in place learning was attributable to motor or sensory impairment, all mice were given a visible platform test. The results showed no significant difference between transgenic and non-transgenic mice for all age groups (data not shown).

ChAT activities

The Table shows the results of ChAT assays in the brains of both transgenic and non-transgenic mice at 3, 6 and 9 months of age. A significant decrease ($P < 0.05$) in ChAT activities was observed in both the cortex and hippocampus of 9-month-old transgenic mice relative to those of the age-matched non-transgenic mice. Such changes were not found in the transgenic mice at either 3 or 6 months of age. ChAT levels in the hypothalamus and corpus striatum were also measured, and no significant differences were observed for any of age groups (data not shown).

Table. ChAT activity levels in four brain regions of the transgenic and non-transgenic mice at different ages

Mice	n	ChAT (nmol·h·mg^{-1} protein)			
		Cortex	Hippocampus	Corpus striatum	Hypothalamus
3-month control	8	3.19 ± 0.12	2.02 ± 0.13	9.12 ± 1.01	1.64 ± 0.12
3-month transgenic	8	3.06 ± 0.21	1.93 ± 0.14	9.28 ± 0.86	1.71 ± 0.14
6-month control	8	3.41 ± 0.13	1.91 ± 0.13	9.18 ± 1.25	1.61 ± 0.17
6-month transgenic	8	3.22 ± 0.13	1.90 ± 0.09	9.34 ± 1.36	1.50 ± 0.11
9-month control	8	3.18 ± 0.21	1.74 ± 0.17	9.09 ± 1.14	1.71 ± 0.19
9-month transgenic	8	2.34 ± 0.22*	1.21 ± 0.08*	8.97 ± 0.98	1.70 ± 0.20

* $P < 0.05$, compared with 9-month-old control group.

DISCUSSION

The plus-maze is based on the natural aversion of rodents for an open, high, well-lighted space and is used to measured anxiety in mice. Our results showed that all mice had a consistent preference for the enclosed arms, and the number of total arm entries was statistically similar, indicating that all the mice had a similar degree of axiety.

Spontaneous alternation is considered to be a form of working memory, and impaired function in a Y-maze in mice has been associated with damage to the hippocampal formation. When the transgenic and non-transgenic mice were given a choice of entering either of two arms in a Y-maze, they tended to alternate their choices spontaneously. Spontaneous alternation, however, was significantly lower for 9-month-old transgenic mice, despite a level of total arm entries comparable to the age-matched non-transgenic mice. Further analysis, namely, a corrected alternation score, showed that the spontaneous alternation was not biased by the tendency to alternate or rotate in a favored direction and a position bias. This confirmed the deficits in spontaneous alternation in 9-month-old transgenic mice, but these deficits cannot be attributed to rotation behavior or to a position bias.

The place learning version water maze belongs to a class of mazes for which the normal solution depends on the use of what is called a locale strategy. That is, the place learning version permits the animal to move to a goal object from different starting positions by learning the location of the goal relative to non-maze cues in the environment. Animals that solve this task are considered to have spatial mapping ability. Both the transgenic and non-transgenic mice at either 3 or 6 months of age acquired the place spatial learning readily, as indicated by a decrease in the time taken to reach the platform over sessions. Nine-month-old transgenic mice, however, did not show such a decrease in latency, which could reflect the spatial disorientation. Impairment in performance in the hidden platform task could be affected by other factors such as swimming ability and differences in search strategy. The probe trial results showed that 3-, 6-month mice (both transgenic and non-transgenic mice) and 9-month-old non-transgenic mice were searching selectively the target quadrant of the maze and crossing the exact former platform location more than comparable areas in the other three quadrants. However, 9-month-old transgenic mice did not show this selective search strategy. Furthermore, no significant differences were obtained in visible platform learning between transgenic mice and age-matched non-transgenic mice for any age group, suggesting a similar capacity to respond to a visible cue.

The relationship between the central cholinergic system and learning and memory has been the subject of extensive studies.[4] Lesions of the cholinergic neurons of the nucleus basalis deplete the cortex of its cholinergic innervation and cause learning and memory deficits in several animal species.[8] In vivo infusion of synthetic A into the cerebral ventricle of rats induces deficits in water maze performance and a significant reduction in ChAT activities in these rats.[9] This study strongly suggests that Aβ deposition in the brain is related to cholinergic dysfunction and, consequently, cognitive impairment. The present data show that 9-month-old APP_{770} transgenic mice exhibit remarkable deficits in both spontaneous alternation in a Y-maze, a task thought to involve the hippocampus, and spatial learning in a water maze. Such deficits were not observed in either 3- or 6-month-old transgenic mice. ChAT activities were also decreased significantly, particularly in the cortical and hippocampal regions, in the 9-month-old transgenic mice, but remained unchanged in the transgenic mice at 3 or 6 months of age. Whether the learning and memory deficits in these mice are caused by or merely correlate with a decrease in brain ChAT levels remains unresolved.

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